BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

INTER LIBRARY LOAN REQUEST FORM

RECEIVED
FEB 29 2000
TECH/CHEM. DIVISION (5110)

Borrower's Name	JANET KERR	Org. or A.U.	1633	Phone	5-4055
Serial Number	09/390,634	Date of Request	2/29/00	Date Needed By	Soon please

Please Attach Copy Of Abstract, Citation, Or Bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form.

Author/Editor:	DeVitry et al.
Journal/Book Title:	Neurochem. Int.
Article Title:	Partial Expression of monoaminergic ...
Volume (Issue):	9(1)
Pages:	43-54
Year of Publication:	1986
Publisher:	
Remarks:	
	Dx - sNo

Y, NO
3/1

STIC Use Only

Accession Number: 28497

LIBRARY ACTION	LC		NAL		NIH		NLM		NBS		PTO		OTHE	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

~~Orders 3/2~~
VRC 3/2

Remarks/Comm
1st & 2nd denot
time taken to a
library

O/N - Under NL
means Overnigh
Service

FX - Means Fax
us

Neurochem. Int. Vol. 9, No. 1, pp. 43–53, 1986
Printed in Great Britain

0197-0186/86 $3.00 + 0.00
Pergamon Journals Ltd

PARTIAL EXPRESSION OF MONOAMINERGIC (SEROTONINERGIC) PROPERTIES BY THE MULTIPOTENT HYPOTHALAMIC CELL LINE F7. AN EXAMPLE OF LEARNING AT THE CELLULAR LEVEL

F. DE VITRY[1]*, J. CATELON[1], M. DUBOIS[2], J. THIBAULT[3], D. BARRITAULT[4], J. COURTY[4], S. BOURGOIN[5] and M. HAMON[5]

[1]Neuroendocrinologie Cellulaire et Moléculaire, Collège de France, 11 Place Marcelin Berthelot, 75231 Paris, Cedex 05, France

[2]INRA, Physiologie de la Reproduction, 37000 Nouzilly, France

[3]Neurobiologie Cellulaire et Moléculaire, Collège de France, 11 Place Marcelin Berthelot, 75231 Paris, Cedex 05, France

[4]Biotechnologie, Université Paris Val-de-Marne, 94010 Créteil, France

[5]INSERM U. 288—Neurobiologie Cellulaire et Fonctionnelle, Faculté de Médecine Pitié Salpétrière, 91 Boulevard de l'Hôpital, 75634 Paris, Cedex 13, France

(*Received* 2 *July* 1985; *accepted* 3 *December* 1985)

Abstract—A serum-free medium supplemented with a glial conditioned medium, a brain extract from 8- to 10-day-old mice, hormones, and eye-derived growth factor has been devised which permitted the mouse primitive hypothalamic nerve cell line F7 to express some biochemical properties typical of monoaminergic neurons. Maximal expression was obtained when the culture conditions were applied for 2 days. Most (90–95%) cells then synthesized [^{3}H]serotonin from [^{3}H]5-hydroxytryptophan (but not from [^{3}H]tryptophan). No synthesis was detected in the presence of carbidopa (20 μM), therefore suggesting the involvement of L-aromatic-amino-acid decarboxylase in this process. In addition, F7 cells cultured in such serum-free medium exhibited the capacity of accumulating exogenous serotonin by an ouabain-sensitive mechanism. These data further supported that active molecules in the cell environment can induce, in a primitive cell line, some of the enzymatic activities associated with monoaminergic neurons. Since other well-defined culture conditions can promote the differentiation of the same clone into oligodendrocytes (De Vitry *et al.*, 1983), it can be concluded that the F7 cell has the properties of an embryonic stem cell of the CNS which, depending on external signals, may switch into different alternative developmental neural pathways. We postulate that the stabilization of neuron-like properties due to repetitive cell stimulation by active signals in the environment may represent an example of learning at the cellular level.

INTRODUCTION

Determination of neurochemical specificity is one of the major problems underlying brain development. Although much is known about the features of differentiated neurons, little information has been obtained so far concerning the molecular mechanisms which underlie their emergence during development of the central nervous system (CNS). Cells of the ventricular zone are considered to be the ultimate progenitors of all cell types in the adult CNS (Boulder Committee, 1970). They divide rapidly and after retiring from their last mitotic cycle, neurons detach and migrate towards their final position.

In the mouse, the hypothalamus begins its development on the 10th day of gestation, soon after the closure of the neural tube, and the majority of hypothalamic neurons cease to divide between embryonic day (ED) 12 and ED 14 (Niimi *et al.*, 1962). Before ED 16, most neurons in the mouse are already grouped into several hypothalamic nuclei (Niimi *et al.*, 1962; Shimada and Nakamura, 1973). Neuronal characteristics, such as the presence of $\gamma\gamma$-enolase, have been shown to be already present in some ventricular cells before their migration (De Vitry *et al.*, 1980).

*To whom all correspondence should be addressed.

Studies on the early differentiation steps of hypothalamic neurons are hampered by several problems. One is that critical events take place in very small populations of cells; another arises from the fact that major processes of differentiation take place in early stages, that are still largely inaccessible to experimental manipulations *in vivo*. A possible way to get round some of these difficulties is to use stem cell lines as a model system for looking at certain aspects of differentiation *in vitro*. The hypothalamic cell line F7, initially obtained by viral transformation of ED 14 mouse embryos, has been shown to exhibit properties of a primitive nerve cell and potentialities of a pluripotent cell (De Vitry, 1977). We have devised previously a serum-free medium which permits the induction of oligodendroglial differentiation of this primitive cell (De Vitry *et al.*, 1983). Alternatively, after transplantation into mice, F7 cells can switch to neuronal cells synthesizing neurophysin (De Vitry, 1977).

In the present study, we show that under appropriate conditions which may mimick, at least partly, those found during early brain development, the F7 cell line acquires some of the properties typical of serotoninergic neurons, such as the capacity of synthesizing serotonin (5-HT) from 5-hydroxytryptophan (5-HTP), and of accumulating exogenous 5-HT by an active uptake process (Osborne, 1982). To our knowledge, this is the first time that a progenitor cell of the CNS has been induced to express—however partially—the properties of differentiated neurons, e.g. those using monoamines as neurotransmitters, under controlled environmental conditions. We postulate that such a commitment (and/or stabilization) of a cellular program represents an example of learning at the cellular level.

EXPERIMENTAL PROCEDURES

Materials

Bovine insulin, human transferrin, progesterone, triiodothyronine (T^3), corticosterone, retinol, N^2,O^6-dibutyryl cyclic adenosine monophosphate (dibutyrylcyclic AMP), ethanolamine and ouabain were purchased from Sigma (St Louis, Mo., U.S.A.). Synthetic human parathyroid hormone (sequence 1–34) was generously given by Dr Mouptard (Hôpital St Antoine, Paris, France).

Other drugs were: *d*-lysergic acid diethylamide (LSD, Sandoz, Basel, Switzerland), pargyline (Abbott, Chicago, U.S.A.), *p*-chlorophenylalanine methylester (pcpa, Labkemi, A.B., Göteborg, Sweden), carbidopa, reserpine (Ciba-Geigy, Basel, Switzerland), fluoxetine (Eli Lilly & Co, Indianapolis, U.S.A.) and citalopram (H. Lundbeck & Co, Copenhagen, Denmark).

The labeled indoles L-tryptophan-[side chain-2,3-^{3}H(N)] ([^{3}H]tryptophan, 11.2 Ci/mmol, New England Nuclear, Boston, Mass., U.S.A.), DL-5-hydroxy-[G-^{3}H]tryptophan ([^{3}H]5-HTP, 5.2–11 Ci/mmol, Amersham International, Amersham, Bucks., U.K.) and [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT, generally labeled, 10.2–15.9 Ci/mmol, Amersham International) were purified by ion-exchange chromatography just before use (Hamon *et al.*, 1974; Héry *et al.*, 1979).

Cell cultures

Serum-containing medium (MS$^+$). Cells were grown in Ham's F10 supplemented with 15% heat-inactivated horse serum (IBF, Paris, France), 2.5% fetal calf serum and 3 mM L-glutamine as previously described (De Vitry, 1977).

Serum-free media (MS$^-$ and MSN). Cells were first seeded at 3–4 × 10^4 cells/35 mm tissue-culture dish (Falcon, Becton Primaria) in serum containing medium MS$^+$ plus F7 conditioned medium (1:1, by vol), and cultured for 24 h at 37°C at saturating humidity in 10% CO_2–90% air atmosphere. Then the medium was changed to one of the following serum-free media: either the "control" medium (MS$^-$) (De Vitry *et al.*, 1983) or the "experimental" medium (MSN) consisting of a mixture of Ham's F12, Dulbecco's modified Eagle medium (DME), and C6 Leibovitz L15-conditioned medium (1:2:1, by vol) (Table 1). The L15-conditioned medium was obtained by cultivating for 3 days confluent C6 glial cells in bottles (75 cm^2) containing 12 ml of L15 and filtering the resulting culture medium through 0.22 μm Millipore filters. The "experimental" MSN medium was supplemented with various hormones and constituents as described in Table 1. All constituents except insulin, transferrin, L-glutamine and C6-conditioned medium, were added every day in 0.4 ml of Ham's F12 and DME (1:2, by vol), replacing 0.4 ml of cultured medium. Steroid hormones, triiodothyronin and retinol were added in an ethanolic solution (1 μl/ml).

Preparation of brain extracts

Brains of Swiss mice at various ages (adult, 8–10 days postnatal, neonatal or ED 18) were dissected and homogenized in 5 vol (w/v) of ice-cold saline buffered with 10 mM Na^+ phosphate (pH 7) using a Polytron disrupter. Homogenates were centrifuged at 105,000 *g* for 30 min at 4°C, and an aliquot (0.1 ml) of supernatants was added to culture dishes each day.

Preparation of eye-derived growth factor (EDGF)

EDGF was prepared from bovine retina, as described elsewhere (Barritault *et al.*, 1982). The fraction used was the "acetic acid purified EDGF" (Barritault *et al.*, 1982). Maximal inductive effect was obtained by adding 15 μl of EDGF (1000 stimulating units/ml) to each culture dish (total volume: 2 ml).

Immunocytochemical techniques

The antiserum presently used was raised in a rabbit with 5-HT coupled to bovine serum albumin (BSA) by formaldehyde, according to the procedure of Steinbusch *et al.* (1978). The presence of 5-HT antibodies was attested by the positive precipitin ring test and micro-complement fixation (Wasserman and Levine, 1961) using 5-HT coupled to ovalbumin (without cross-reaction with BSA) by formaldehyde as the antigen. Furthermore an ELISA test (Voller *et al.*, 1979) was developed by binding the antiserum (dilution 1/16,000) to polystyrene tubes coated with 5-HT–ovalbumin. Using this test, ovalbumin was totally inactive, 6.4 ng of 5-HT–ovalbumin (corresponding to 1 nM

Table 1. Composition of media

	MS$^+$
Ham's F10:F7-conditioned me	
L-Glutamine, 3 mM	
Horse serum, 15%	
Fetal calf serum, 2.5%	

Cells were first seeded in MS$^-$ resulted in a reduction of
+DME = Dulbecco's modifiec
±SU = stimulating unit.

5-HT) gave half displac
antigen and 0.5 μM of u
produce the same half
5-methyl-tryptamine and
active in the micromola
but tryptophan, 5-HTP,
indole acetic acid, tryp
were totally inactive at
description of the spe
presently used has beer
Dubois, 1985).

For the immunocytoc
tured cells, the indirect
used with minor modifica
on plastic dishes were
pargyline and then for
mentioned, ouabain (0.5
as pargyline. Cells were
for 2 h in 6% neutral f
containing 1 mM MgSO
(pH 7.4). After a further
were subsequently expo
15–17 h at 4°C (1:400 f
for the complete inacti
for 30 min with Na^+ pl
anti-rabbit sheep immu
peroxidase (Institut Pas
activity was then reve
benzidine as described
specificity of the reacti
rabbit serum, as well
adsorbed by an excess
of 1 mM).

Incubation with [^{3}H]tr

[^{3}H]Tryptophan (2
[^{3}H]5-HTP (1.5–2.6 μ
the culture medium (2

Table 1. Composition of media used for culturing F7 cells: serum-containing medium (MS^+), serum-free medium (MS^-) and serum-free medium devised for expression of neuronal properties (MSN)

MS^+	MS^-	MSN
Ham's F10:F7-conditioned medium	Ham's F12:DME† (1:3, by vol)	Ham's F12:DME†, *C6 L15-conditioned medium (1:2:1, by vol)
L-Glutamine, 3 mM	Insulin, 10 μg/ml	Insulin, 10 μg/ml
Horse serum, 15%	Transferrin, 25 μg/ml	Transferrin, 25 μg/ml
Fetal calf serum, 2.5%	L-Glutamine, 2.4 mM	L-Glutamine, 2.4 mM
	Parathyroid hormone, 2 ng/ml	Parathyroid hormone, 4 ng/ml
	Glucose, 10 mM	Ethanolamine, 0.1 mM
		*D(+)Galactose, 10 mM
		*L-Tyrosine, 10 nM
		*L-Tryptophan, 10 nM
		*5-HTP, 0.1 μM
		*Progesterone, 1 nM
		*Corticosterone, 10 pM
		*Triiodothyronin, 10 pM
		*Retinol, 1 nM
		*Lys-vasopressin, 2.5 IU/ml
		*Dibutyryl cyclic AMP, 100 μM
		*EDGF, 7.5 SU‡/ml
		*8- to 10-day-old mice brain extract, 50 μl/ml
		*LSD, 10 μM

Cells were first seeded in MS^+ and cultured for 24 h. Then the medium was changed to MS^- or MSN. Deletion of any single* constituent resulted in a reduction of cell attachment, survival and induction of 5-HT uptake capacity.
†DME = Dulbecco's modified Eagle medium.
‡SU = stimulating unit.

5-HT) gave half displacement of the specifically bound antigen and 0.5 μM of uncoupled 5-HT was necessary to produce the same half displacement. Free tryptamine, 5-methyl-tryptamine and 5-methoxytryptamine were also active in the micromolar range ($4\ \mu M < IC_{50} < 12\ \mu M$), but tryptophan, 5-HTP, 5-methoxytryptophan, 5-hydroxyindole acetic acid, tryptophol and 5-hydroxytryptophol were totally inactive at least up to 0.1 mM. A detailed description of the specificity of the 5-HT antiserum presently used has been reported elsewhere (Tillet and Dubois, 1985).

For the immunocytochemical detection of 5-HT in cultured cells, the indirect immunoperoxidase technique was used with minor modifications (De Vitry, 1977). Cells grown on plastic dishes were treated for 30 min with 10 μM pargyline and then for 30 min with 5-HT (20 μM). When mentioned, ouabain (0.5 mM) was added at the same time as pargyline. Cells were then rapidly rinsed and fixed at 4 C for 2 h in 6% neutral formaldehyde or paraformaldehyde containing 1 mM $MgSO_4$ in 10 mM Na^+ phosphate buffer (pH 7.4). After a further rinse for 2 h in the same buffer, cells were subsequently exposed to the specific antiserum for 15 17 h at 4°C (1:400 final dilution containing 0.1% BSA for the complete inactivation of BSA antibodies), washed for 30 min with Na^+ phosphate buffer, and incubated with anti-rabbit sheep immunoglobulins coupled to horseradish peroxidase (Institut Pasteur; 1:80 final dilution). Peroxidase activity was then revealed with H_2O_2 and 3,3'-diaminobenzidine as described previously (De Vitry, 1977). The specificity of the reaction was tested by using preimmune rabbit serum, as well as the specific antiserum previously adsorbed by an excess of 5-HT (at a final concentration of 1 mM).

Incubation with [^{3}H]tryptophan or [^{3}H]5-HTP

[^{3}H]Tryptophan (2.4–3.6 μM final concentration) or [^{3}H]5-HTP (1.5–2.6 μM final concentration) was added to the culture medium (2 ml/dish) immediately after transferring cells to serum-free conditions, and then added each day thereafter. For these experiments, the corresponding "cold" precursor compounds (see Table 1) were omitted. On the last day of incubation, 0.2 ml of 4 N $HClO_4$ were added to each culture dish 2 h after supplying the medium with the radioactive precursor. Cell lysate was collected and then pooled with a further wash of the culture dish with 0.8 ml of 0.4 N $HClO_4$. The mixture was supplemented with 5 mM ascorbic acid and 2 μg 5-HT, and adjusted to pH 6.5 with 0.2 M KH_2PO_4/K_2HPO_4 (pH 5.5) containing 7.5% KOH (w/v). After centrifugation (8000 *g*, 15 min at 4°C), each clear supernatant was then passed through a microcolumn (1.3 cm high, 0.2 cm dia) of Dowex AG 50 WX4 buffered at pH 6.5 with 0.2 M Na^+ phosphate. The subsequent steps were then as described in detail by Héry *et al.* (1979). Briefly, [^{3}H]5-HT adsorbed onto the Dowex column was eluted with 1 ml of a 2 N $HClO_4$/ethanol solution (1:2, v/v) and the eluate was neutralized with 0.2 M KH_2PO_4/K_2HPO_4 (pH 5.5) containing 7.5% KOH. The $KClO_4$ precipitate was discarded by centrifugation and [^{3}H]5-HT in the clear supernatant was adsorbed onto an Amberlite (CG50) column (1.5 cm high, 0.4 cm dia). [^{3}H]5-HT was finally eluted with 2 ml of 0.5 M Na^+ borate buffer (pH 10) and estimated by liquid scintillation counting of an aliquot (1 ml) of the eluate mixed with 10 ml of Aquasol™ (New England Nuclear).

Under such conditions, the radioactivity finally recovered in "blank" samples (starting with 20–30 μCi pure [^{3}H]tryptophan or [^{3}H]5-HTP) was about 100 cpm, i.e. 0.1 nCi. The spectrophotofluorimetric estimate of "cold" 5-HT added to each sample indicated an average recovery of 60% for the indoleamine (Héry *et al.*, 1979).

Incubations with [^{3}H]5-HT

Cells were maintained under various conditions (MS^+, MS^-, MSN) for 2 days before the addition of 1.0 μCi of purified [^{3}H]5-HT (final concentration: 30 nM). The incubation was stopped 30 min later by the addition of 0.2 ml of

4 N $HClO_4$ to each culture dish, and [^{3}H]5-HT was then extracted as described for the experiments with ^{3}H-labeled precursors. In addition, the effluents and washing of the Dowex microcolumns were further chromatographed onto Sephadex G10 columns in order to detect the possible formation of [^{3}H]5-HIAA: details of the protocol used have been reported elsewhere (Hamon *et al.*, 1973). Blanks consisted of samples incubated under exactly the same conditions except that no cells were seeded in the culture dishes. Such controls indicated that no spontaneous degradation of [^{3}H]5-HT occurred for the 30 min incubation period.

Identification of [^{3}H]5-HT

Amberlite eluates were neutralized with 3 N HCl, and aliquots (0.1 ml) were submitted to HPLC on an Ultrasphere IP column (25 cm long, 0.46 cm dia, 5 μm) using a mobile phase (flow rate = 1 ml/min) consisting of 70 mM K^+ phosphate, 2 mM triethylamine, 0.1 mM EDTA and 12% methanol, adjusted to pH 5.1 with citric acid. The elution of 5-HT was monitored by electrochemical detection at 0.65 V. The column eluate was collected as successive 0.5 ml fractions which were mixed with 10 ml Aquasol™ for radioactivity counting.

Determination of met-enkephalin content

Cells of 20 tissue culture dishes grown for 2 days in the presence (MS^+) or the absence (MSN) of serum were collected, rinsed twice with 0.1 M Na^+ phosphate buffer (pH 7.4) containing 0.9% NaCl, and sonicated in 0.1 N HCl. Their met-enkephalin content was then measured using a specific radioimmunoassay (Cesselin *et al.*, 1982). The reliable sensitivity of this assay is 0.1 pg.

Proteins were determined using the Folin phenol procedure (Lowry *et al.*, 1951) with BSA (Sigma) as the standard.

Statistical analysis

Differences were considered as being statistically significant when the Student's t-test gave a P-value <0.05.

RESULTS

Morphology of the clonal cell line grown in the presence or absence of serum

F7 cells exhibited a flat morphology when grown in serum-containing medium (MS^+) (Fig. 1a) (see also De Vitry, 1977).

In MS^- medium, cell growth was approximately the same as in MS^+ medium. However MS^- medium did not permit F7 cells to attach to the plastic dish. Seeding in MS^+ medium was necessary to achieve cell attachment before any experiment could be run in serum-free medium, as already observed for oligodendroglial differentiation of F7 cells in serum-free medium (De Vitry *et al.*, 1983). This led us to culture all cells for 24 h in the MS^+ medium before selecting other culture conditions (see Experimental Procedures).

In contrast to F7 cells grown in MS^+ or MS^- medium, those grown in the serum-free medium MSN supplemented with hormones, 8- to 10-day-old mice brain extract, and EDGF, became bipolar or multipolar with extended short processes, provided seeding of cells was done at low density (3.4×10^4 cells/dish) (Fig. 1b).

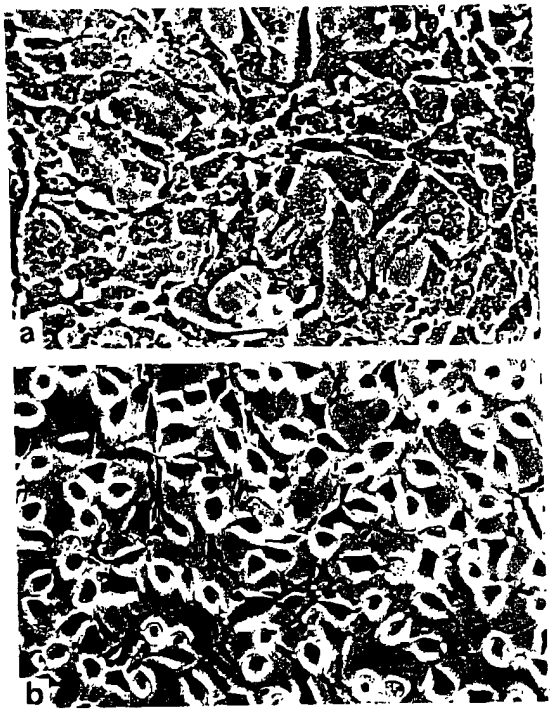

Fig. 1. F7 cells: phase contrast. Cells grown in: (a) MS^+ medium, (b) MSN medium; ×320.

Immunohistochemical evidence of 5-HT uptake capacity in F7 cells cultured in MSN medium

In contrast to F7 cells grown in the presence of serum (MS^+) or in basal serum-free medium (MS^-) which gave no positive staining, those maintained for 2 days in MSN medium exhibited a marked immunoperoxidase staining for 5-HT provided they were preincubated for 30 min with 20 μM of the indoleamine. Staining appeared to be mainly concentrated at one or two poles of the cytoplasm near the surrounding cytoplasmic membrane (Fig. 2a). In contrast, no positive immunocytochemical reaction was noted in F7 cells which kept the flat morphology typical of MS^+ or MS^- culture conditions; usually such flat cells represented <5% of the total cell population under MSN culture conditions. As expected for a specific staining, all cells remained uncolored when the immunocytochemical reaction was carried out with a preimmune serum or with

Fig. 2. Immunocytochem
clonal cell line grown in M
for 30 min with 20 μM
5-HT antiserum; (b) co
viously adsorbed by 5-H
treatment of cell

the 5-HT antiserum
indoleamine (Fig. 2b).

As illustrated in Fig.
observed when ouabai
culture medium 30 mi
contrast, specific inhibi
serotoninergic neuron
(Wong *et al.*, 1983) o
1982) did not prevent
genous indoleamine in
conditions.

Fig. 2. Immunocytochemical detection of 5-HT in the F7 clonal cell line grown in MSN medium. Cells were incubated for 30 min with 20 μM 5-HT before fixation (×480). (a) 5-HT antiserum; (b) control with 5-HT antiserum previously adsorbed by 5-HT; (c) 5-HT antiserum after 1 h treatment of cells with 0.5 mM ouabain.

the 5-HT antiserum previously adsorbed with the indoleamine (Fig. 2b).

As illustrated in Fig. 2c, uncolored cells were also observed when ouabain (0.5 mM) was added to the culture medium 30 min before 5-HT (20 μM). In contrast, specific inhibitors of 5-HT uptake in central serotoninergic neurons such as 10 μM fluoxetine (Wong *et al.*, 1983) or 10 μM citalopram (Hyttel, 1982) did not prevent the accumulation of the exogenous indoleamine in F7 cells under MSN culture conditions.

Synthesis of [^{3}H]5-HT from [^{3}H]5-HTP but not from [^{3}H]tryptophan in F7 cells cultured in MSN medium

In contrast to cells cultured in the presence of serum (MS$^+$), those maintained for 2 days in MSN medium were able to convert [^{3}H]5-HTP into [^{3}H]5-HT (Table 2). Identification of [^{3}H]5-HT as the final product was made by ion-exchange chromatography and HPLC analysis, as shown in Fig. 3. Further confirmation of the synthesis of the indoleamine [^{3}H]5-HT was obtained by the addition of 20 μM carbidopa into the culture medium since the resulting blockade of L-aromatic-amino-acid decarboxylase (AADC) activity was associated with the loss of cell capacity to decarboxylate [^{3}H]5-HTP (Table 2). In contrast, monoamine oxidase inhibition by 10 μM pargyline did not affect the [^{3}H]5-HT content in culture dishes supplemented with [^{3}H]5-HTP for 3 days (Table 2, see Experimental Procedures). The lack of monoamine oxidase activity in F7 cells was confirmed using exogenous [^{3}H]5-HT: no significant conversion of [^{3}H]5-HT into [^{3}H]5-hydroxyindole acetic acid ([^{3}H]5-HIAA) occurred for a 30 min incubation of F7 cells with the [^{3}H]indoleamine. Similar negative findings were ob-

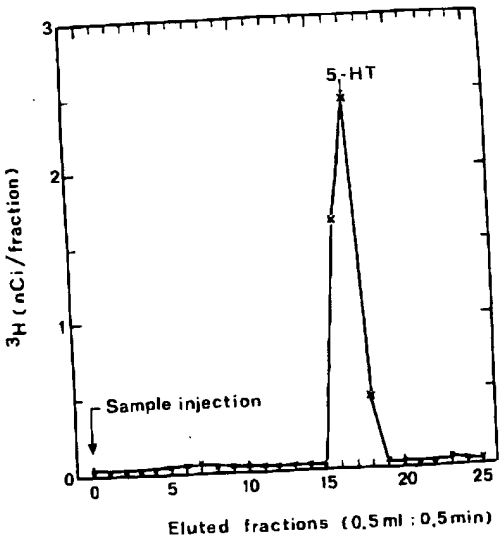

Fig. 3. HPLC elution profile of [^{3}H]5-HT synthesized from [^{3}H]5-HTP by F7 cells grown in MSN medium. Cells were grown for 2 days in MSN medium (2 ml) supplemented with 13 μCi [^{3}H]5-HTP, and newly synthesized [^{3}H]5-HT was extracted as described in Experimental Procedures. The HPLC elution profile of [^{3}H]5-HT was established by counting the radioactivity of each collected fraction (0.5 ml). The elution volume of authentic 5-HT (8.5 ml) was monitored by electrochemical detection (E = 0.65 V). Similar results were obtained in 5 independent experiments.

Table 2. Differential use of [^{3}H]5-HTP, [^{3}H]tryptophan and [^{3}H]5-HT by F7 cells cultured under various conditions

Added substrate	Culture conditions	[^{3}H]5-HT (nCi)
[^{3}H]5-HTP (16 μCi)	MS$^+$	ND
	MSN	51.61 ± 4.02
	MSN + carbidopa (20 μM)	0.46 ± 0.09
	MSN + pargyline (10 μM)	58.86 ± 5.31
[^{3}H]tryptophan (20 μCi)	MS$^+$	ND
	MSN	ND
[^{3}H]5-HT (1.0 μCi)	MS$^+$	936 ± 60
	MSN	948 ± 72

In all cases, F7 cells were grown for 24 h in the presence of serum (MS$^+$) to allow their attachment to the culture dishes, and then for 48 h either in the same medium (MS$^+$) or in the serum-free medium supplemented with inducing factors (MSN). [^{3}H]5-HTP or [^{3}H]tryptophan was added each day up to a total content of 16–20 μCi/culture dish (2 ml of medium). [^{3}H]5-HT was added 30 min before the addition of 0.2 ml of 4 N $HClO_4$ to stop the reaction (see Experimental Procedures). The total content of [^{3}H]5-HT was measured in each case and expressed as nCi/dish. Each value is the mean ± SEM of 6 separate determinations. ND = not detectable.

tained with cells cultured in the presence (MS$^+$) or absence (MS$^-$, MSN) of serum.

As shown in Table 2, no synthesis of [^{3}H]5-HT was found when [^{3}H]tryptophan was used as the labeled substrate in any of the culture conditions presently studied (MS$^+$ or MSN medium).

Effects of reserpine treatment or K$^+$-induced depolarization on the accumulation of [^{3}H]5-HT newly synthesized from [^{3}H]5-HTP in cultured F7 cells

After a 2-day culture in MSN medium supplemented with [^{3}H]5-HTP, newly synthesized [^{3}H]5-HT was found principally in the culture medium. Indeed only 1.5% of total [^{3}H]5-HT was recovered in the cell pellet (Fig. 4). The addition of 1 μM reserpine to the culture medium 1 h before collecting cells by centrifugation did not alter their [^{3}H]5-HT content. In contrast, depolarization by an excess of K$^+$ (40 mM KCl added 1 h before the centrifugation) resulted in a significant decrease (−36%) of the cellular accumulation of newly synthesized [^{3}H]5-HT (Fig. 4).

Respective involvement of various MSN constituents for the induction of 5-HT uptake and synthesizing capacities in F7 cells

The effect of hormones and factors on both culture survival and 5-HT uptake and synthesizing capacities was investigated with serum-free medium in which the concentrations of one or two hormones or growth factors were systematically altered compared to the complete MSN medium.

Deletion of any single constituent resulted in a reduction of 5-HT uptake in F7 cells. This is illustrated in Fig. 5 which shows a marked decrease in the immunostaining of F7 cells preincubated with 5-HT when LSD was omitted from the cultured medium. Similar pictures were obtained when any compound labeled with an asterisk in Table 1 was omitted.

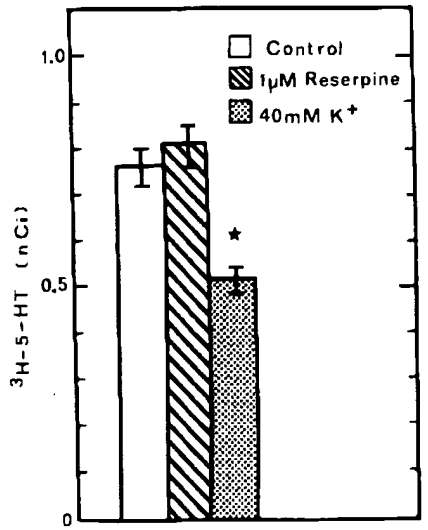

Fig. 4. Effect of reserpine treatment or K$^+$-induced depolarization on the [^{3}H]5-HT cellular content of F7 cells grown in MSN medium supplemented with [^{3}H]5-HTP. F7 cells were grown for 2 days in MSN medium (2 ml) containing 18 μCi of [^{3}H]5-HTP and then exposed for 1 h to 1 μM reserpine or 40 mM K$^+$. After collection from the culture dishes, cells were centrifuged and the corresponding pellet was washed once with Na$^+$ phosphate buffer. Finally, [^{3}H]5-HT was extracted with 0.4 N $HClO_4$ and estimated as described in Experimental Procedures. Each bar is the mean ± SEM of 4 independent determinations of [^{3}H]5-HT (in nCi) found in the cell pellets. The total content of [^{3}H]5-HT newly synthesized from [^{3}H]5-HTP was not significantly affected by either treatment ($\bar{X} = 44.5 \pm 3.8$ nCi, $n = 12$). *$P < 0.05$ when compared to respective controls.

Fig. 5. Effect of LSD deletion from the MSN culture medium on 5-HT uptake capacity of F7 cells (×480). Cells were preincubated for 30 min with 20 μM 5-HT in MSN medium before fixation and immunostaining with the 5-HT antiserum: (a) MSN, (b) MSN without LSD. Note a marked decrease in the immunostaining of F7 cells in the absence of LSD.

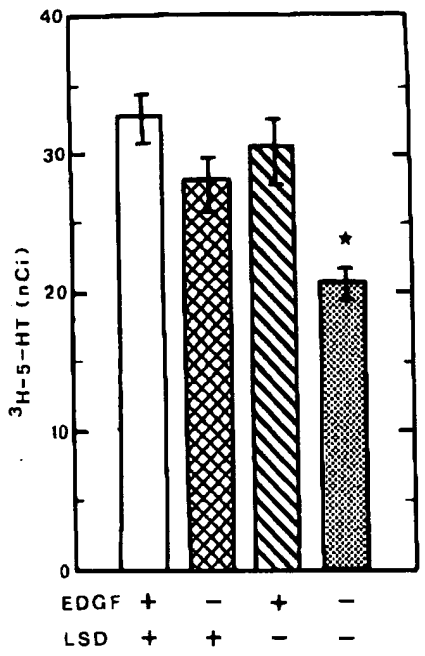

Fig. 6. Effects of EDGF and/or LSD deletion from the MSN culture medium on the synthesis of [^{3}H]5-HT from [^{3}H]5-HTP by F7 cells. F7 cells were grown for 2 days either in complete MSN medium or in the same medium deprived of EDGF (15 μl/2 ml) and/or LSD (10 μM) as indicated on the abscissa. The same amount of [^{3}H]5-HTP (10 μCi) was added to all culture dishes and newly synthesized [^{3}H]5-HT was then estimated as described in Experimental Procedures. Each bar is the mean ± SEM of 4 independent determinations. $*P < 0.05$ when compared to [^{3}H]5-HT synthesis in complete MSN medium (empty bar).

Studies of a wide range of concentrations of each compound led to the MSN medium (see the composition in "Table 1") allowing optimal cell attachment and survival, and induction of 5-HT uptake capacity.

One critical factor was the age of the mice from which brain extracts were prepared. Thus, cultures with extracts from ED 18, newborn, 5-day-old, 8- to 10-day-old or adult (~90-day-old) mice revealed that the induction of 5-HT uptake capacity was maximal with extracts from 8- to 10-day-old animals.

Similar experiments conducted to identify which factor(s) was (were) responsible for the induction of [^{3}H]5-HT synthesizing capacity indicated that complex interactions of several factors probably played much more important roles than any individual factor. A typical example of such an interaction is illustrated in Fig. 6. Although the removal of LSD or EDGF alone exerted only a discrete influence on the capacity of F7 cells to synthesize [^{3}H]5-HT from [^{3}H]5-HTP, the simultaneous removal of both compounds resulted in a marked decrease in this capacity (Fig. 6).

Time-dependent irreversible commitment of F7 cells to take up 5-HT under MSN culture conditions

To determine whether the appearance of 5-HT uptake capacity represented an irreversible commitment towards a monoaminergic lineage or not, F7 cells were first grown for 1 or 2 days in MSN medium and then switched to serum-supplemented culture conditions (MS^+).

Most F7 cells grown in MSN medium for 2 days were then unable to survive in MS^+ medium. Only the flat cells representing 3–5% of the total cell population remained attached to the dish and went on dividing. However, cells that were grown for only 1 day in MSN medium remained attached and survived after the switch to MS^+ medium, but they lost their capacity to accumulate exogenous 5-HT.

Table 3. Met-enkephalin-like material (MELM) in F7 cells cultured in the presence (MS^+) or absence (MSN) of serum

Culture conditions	MELM (pg/mg protein)
MS^+	5.04 ± 1.15
MSN	3.86 ± 0.53

F7 cells were grown for 24 h in MS^+ medium and then for 48 h either in the same medium or in serum-free medium supplemented with inducing factors (MSN). MELM content of F7 cells was then estimated by radioimmunoassay (Cesselin *et al.*, 1982) and expressed as pg met-enkephalin equivalents/mg protein. Each value is the mean ± SEM of 3–4 independent determinations.

Met-enkephalin content in F7 cells cultured in the presence or absence of serum

In agreement with previous data (Cesselin *et al.*, 1982), we detected met-enkephalin-like immunoreactivity in F7 cells cultured in MS^+ medium (Table 3). In contrast to 5-HT uptake and synthesizing capacities, the levels of met-enkephalin-like material in F7 cells did not depend upon the culture conditions: similar levels were found in F7 cells grown in MS^+ or MSN medium (Table 3).

DISCUSSION

The purpose of this investigation was to study the possible commitment of a pluripotent nerve cell line towards a neuronal lineage, and shed some light on the environmental signals involved in this determination. The hypothalamic cell line F7 (De Vitry, 1977) was used here as a model for such pluripotent nerve stem cells in the CNS.

A preliminary step consisting of defining a serum-free medium able to support proliferation of F7 cells for short duration experiments has been previously achieved (De Vitry *et al.*, 1983). On account of the report by Bottenstein and Sato (1979) that neuronal cell lines could survive *in vitro* in the presence of specific combinations of hormones instead of serum, and our own results showing that oligodendroglial differentiated properties could be induced in F7 cells by appropriate serum-free culture conditions (De Vitry *et al.*, 1983), we then decided to pursue our investigations with the development of a serum-free medium which would promote the induction of neuronal properties in primitive nerve cells.

In contrast to F7 cells cultured in the presence of serum, those maintained in this specially devised medium MSN (Table 1) were able to accumulate exogenous 5-HT, and synthesize [^{3}H]5-HT from [^{3}H]5-HTP but not from [^{3}H]tryptophan, showing the presence of AADC (EC 4.1.1.28) but not of tryptophan hydroxylase. By immunocytochemistry, F7 cells with newly acquired 5-HT uptake capacity were readily identifiable in culture 2 days after their transfer into serum-free medium (MSN), provided they were seeded at low density. Instead of the typical flat morphology observed in the presence of serum, F7 cells cultured in MSN medium exhibited a bipolar or multipolar shape with several extended processes which gave them neuron-like patterns.

In spite of these newly acquired properties, F7 cells could not be considered as serotoninergic neurons since they lacked the specific enzyme tryptophan hydroxylase. Furthermore, the uptake process responsible for 5-HT accumulation in F7 cells cultured in MSN medium was inhibited by the Na^+ pump blocker ouabain, as expected from a functional coupling between Na^+/K^+-dependent ATPase and the 5-HT carrier, like in serotoninergic neurons (Bogdanski *et al.*, 1970), but not by typical 5-HT uptake blockers such as fluoxetine and citalopram (Wong *et al.*, 1983; Hyttel, 1982).

Other monoaminergic neurons such as dopaminergic, noradrenergic and adrenergic neurons also contain the enzyme AADC and exhibit some ability to take up 5-HT (Shaskan and Snyder, 1970). Accordingly it could be proposed that F7 cells cultured in MSN medium were in fact more catecholaminergic than serotoninergic. However immunostaining with anti-tyrosine hydroxylase antiserum (Thibault *et al.*, 1981) was negative, and no ^{3}H-labeled catecholamine was found after a 3-day culture in MSN medium supplemented with [^{3}H]tyrosine. In contrast, these cells did exhibit the capacity to convert [^{14}C]dihydroxyphenylalanine ([^{14}C]DOPA) into [^{14}C]dopamine (unpublished observations), as expected from their expression of AADC.

In addition to well-characterized monoaminergic neurons, other cells have been shown to contain AADC but apparently neither tryptophan hydroxylase nor tyrosine hydroxylase in the CNS (Jaeger *et al.*, 1983, 1984). These cells may correspond at least partly to the APUD cells characterized by Pearse (1969) since they Decarboxylate the Amine Precursors (5-HTP or DOPA) which has been taken Up. Whether F7 cells cultured in MSN medium belong to the APUD lineage remains an interesting possibility since, like APUD cells, they not only decarboxylate 5-HTP or DOPA but also contained a neuropeptide, namely met-enkephalin (Cesselin *et al.*, 1982; Table 3 of the present paper). Furthermore, Teitelman *et al.* (1983) noted that cells expressing AADC appear very early in the notochord and the neural tube of developing embryo, well before catecholaminergic neurons, and therefore AADC-containing F7 cells

sently studied, which derive from the mouse *em-*, might well belong to this cell type. Alterna-ly, they might correspond to some precursors of onoaminergic neurons since it has been proposed AADC is expressed before tyrosine hydroxylase tryptophan hydroxylase) in such neurons telman *et al.*, 1983). More studies are needed to rmine if F7 cells really express a preestablished etic program, due for instance to their hypo-lamic origin, or whether the lack of interactions th other environmental cues is responsible for absence of tryptophan hydroxylase or tyrosine droxylase.

The possible relationship between the acquisition F7 cells of AADC activity and 5-HT uptake pacity, and F7 cell induction to a neuronal or APUD lineage could be further assessed by exam-ning appropriate neuronal markers such as -enolase and the capacity to bind tetanus toxin. However, these two "specific" markers have been found not only in neurons or APUD cells (Schmechel *et al.*, 1978; Schmechel, 1985), but also in some types of glial cells (Raff *et al.*, 1983; Schmechel, 1985). The presence of the "specific" neurofilament triplet proteins in F7 cells cultured in MSN medium is currently under investigation. However, even with these marker proteins, negative findings would not allow negation of F7 cell commitment to a neuronal lineage since typical neurons without noticeable neurofilaments have been already described (Sharp *et al.*, 1982).

The hormones presently chosen for cultivating F7 cells in the absence of serum have already been shown to play major roles for neural development *in vivo* or *in vitro*, e.g. triiodothyronine, corticosterone, progesterone, vasopressin [see Legrand (1983) for a review, and McEwen (1981), Toran-Alleran (1981) and Auerbach and Lipton (1982)]. Other factors included in MSN medium have been selected on the basis of their reported efficacy to promote the differentiation of various cell types; for instance recent investigations have shown that vitamin A and its derivatives (retinoids) induce the *in vitro* differentiation of teratocarcinoma stem cells (Strickland and Mahdavi, 1978; Solter *et al.*, 1979; Jones-Villeneuve *et al.*, 1982). Similarly, dibutyryl cyclic AMP stimulates various differentiation processes in neurons and glial cells (Waymire *et al.*, 1978; Walicke and Patterson, 1981). In addition, the C6 glioma L15-conditioned medium also participated in the induction of AADC and 5-HT uptake capacity in F7 cells cultivated in MSN medium. Previous reports have already mentioned that C6 glioma cells release into the culture medium substances which can support the survival (and process formation) of dissociated neurons from the peripheral nervous system (Barde *et al.*, 1978; Unsicker *et al.*, 1984). A brain extract was another necessary requirement for the induction of 5-HT uptake and synthesizing capacities in F7 cells. While an adult brain extract was essential for oligodendroglial differentiation of F7 cells (De Vitry *et al.*, 1983), only the brain extract from 8- to 10-day-old mice was found in the present study to sustain the expression of 5-HT uptake capacity and AADC activity in F7 cells. Fractionation of these extracts should allow the identification of which factors in the brain of adult or 8- to 10-day-old mice were responsible for the induction of oligodendrocyte or monoaminergic characters in F7 cells, respectively.

It is striking that EDGF, a growth factor activity derived from retina (Barritault *et al.*, 1982), is a necessary—although not sufficient—factor for the induction of both oligodendrocyte (De Vitry *et al.*, 1983) and monoaminergic (this paper) properties of F7 cells. Like NGF (Greene and Tischler, 1976; Schubert *et al.*, 1978), EDGF could act by inducing mitotic arrest and activating a differentiation program; alternatively, it might modify the accessibility of membrane receptors to some external signals. Undoubtedly, EDGF acted upon F7 cells in a different way than on other cell types, since it is generally considered as a factor stimulating cell proliferation but not differentiation (Barritault *et al.*, 1982). Recent developments in the purification of EDGF indicate that it is in fact composed of three biologically active growth factors, two of which are now being purified to homogeneity (Courty *et al.*, 1985). Further investigations will be needed to identify which of these molecules was (were) responsible for the effects observed here.

It has been suggested that monoamines can play a neurotrophic role in early ontogenesis, probably via the stimulation of specific receptors on target cells (Hamon *et al.*, 1985). Some support for this concept is found in the present study since we observed that a 5-HT agonist, LSD [see Hamon (1984) for a review], at least in combination with EDGF, was required for the optimal induction of AADC in F7 cells grown in MSN medium. However, further studies with other 5-HT agonists and antagonists are necessary before the possible involvement of specific 5-HT receptors (on F7 cells) can be proposed, on account of the LSD effect.

In conclusion, the present study revealed that under appropriate culture conditions (MSN), a primitive nerve cell line, F7, previously shown to

differentiate into *oligodendrocytes* under other conditions (De Vitry *et al.*, 1983), could express some of the characteristics typical of monoaminergic *neurons*, e.g. 5-HT uptake capacity and AADC activity. These data provided some support for the possible existence at the early steps of CNS formation of a transitory bipotential cell type which can give rise either to a glial cell (containing specific markers such as carbonic anhydrase II and myelin basic protein) or a neuron- or APUD-like cell in response to environmental cues. Since the different factors in MSN medium had to act synergistically and *repetitively* to induce 5-HT uptake and synthesizing capacity in F7 cells, we can postulate that active signals from the cell environment may trigger a preestablished genetic program and stabilize neuronal functions. We define such mechanisms of *reinforcement* and *stabilization* of the commitment of one cell as a process of *learning at the cellular level*. This *learning* could be of general significance for stabilization of nerve cell identity during brain development.

Acknowledgements—This research has been supported by grants from INSERM (CRE 856025) and CNRS (ATP). We are indebted to the pharmaceutical companies for their generous gifts of drugs. We thank C. Pennarun for his skillful technical assistance and Dr H. Gozlan for the HPLC analyses. We are grateful to Dr A. Tixier-Vidal for her encouragements throughout this study.

REFERENCES

Auerbach S. and Lipton P. (1982) Vasopressin augments depolarization-induced release and synthesis of serotonin in hippocampal slices. *J. Neurosci.* **2**, 477–482.

Barde Y. A., Lindsay R. M., Monard D. and Thoenen H. (1978) New factor released by cultured glioma cells supporting survival and growth of sensory neurons. *Nature, Lond.* **274**, 818.

Barritault D., Plouet J., Courty J. and Courtois Y. (1982) Purification, characterization and biological properties of the eye-derived growth factor from retina: analogies with brain-derived growth factor. *J. Neurosci. Res.* **8**, 477–490.

Bogdanski D. F., Tissari A. H. and Brodie B. B. (1970) Mechanism of transport and storage of biogenic amines. III—Effects of sodium and potassium on kinetics of 5-hydroxytryptamine and norepinephrine transport by rabbit synaptosomes. *Biochim. Biophys. Acta* **219**, 189–199.

Bottenstein J. E. and Sato G. H. (1979) Growth of rat neuroblastoma cell line in serum-free supplemented medium. *Proc. natn. Acad. Sci. U.S.A.* **76**, 514–517.

Boulder Committee (1970) Embryonic vertebrate central nervous system: revised terminology. *Anat. Rec.* **166**, 257–262.

Cesselin F., Hamon M., Bourgoin S., Buisson N. and De Vitry F. (1982) Presence of met-enkephalin in a somatostatin-synthesizing cell line derived from the fetal mouse hypothalamus. *Neuropeptides* **2**, 351–369.

Courty J., Loret C., Moenner M., Chevallier B., Lagente O., Courtois Y. and Barritault D. (1985) Bovine retina contains three growth factor activities with different affinit to heparin: eye derived growth factor I, II, III. *Biochim* **67**, 265–269.

De Vitry F. (1977) Growth and differentiation of a primitiv nervous cell line after *in vivo* transplantation into syngen mice. *Nature, Lond.* **267**, 48–50.

De Vitry F., Picart R., Jacque C., Legault L., Dupouey and Tixier-Vidal A. (1980) Presumptive common precu sor for neuronal and glial cell lineages in mouse hypo thalamus. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4165–4169.

De Vitry F., Delaunoy J. P., Thibault J., Buisson N Lamande N., Legault L., Delasalle A. and Dupouey (1983) Induction of oligodendrocyte-like properties in primitive hypothalamic cell line by cholesterol, an ey derived growth factor and brain extract. *EMBO J.* 199–203.

Greene L. A. and Tischler A. S. (1976) Establishment of noradrenergic clonal line of rat adrenal pheochromo cytoma cells which respond to nerve growth factor. *Proc natn. Acad. Sci. U.S.A.* **73**, 2424–2428.

Hamon M. (1984) Common neurochemical correlates to th action of hallucinogens. In *Hallucinogens: Neurochemical Behavioral and Clinical Perspectives* (Jacobs B. L., ed.) pp. 143–169. Raven Press, New York.

Hamon M., Bourgoin S. and Glowinski J. (1973) Feed-back regulation of 5-HT synthesis in rat striatal slices *J. Neurochem.* **20**, 1727–1745.

Hamon M., Bourgoin S., Jaeger J. and Glowinski J. (1974) Effects of LSD on synthesis and release of 5-HT in ra brain slices. *Brain Res.* **69**, 265–280.

Hamon M., Bourgoin S., Chanez C. and De Vitry F. (1986) Do serotonin and other neurotransmitters exert a trophi influence on the immature brain? In *Developmenta Neurobiology* (Guesry P. R., ed.). Raven Press, Ne York. (in press).

Hery F., Simonnet G., Bourgoin S., Soubrie P., Artaud F. Hamon M. and Glowinski J. (1979) Effect of nerv activity on the *in vivo* release of ^{3}H-serotonin continuousl formed from ^{3}H-tryptophan in the caudate nucleus of th cat. *Brain Res.* **169**, 317–334.

Hyttel J. (1982) Citalopram-pharmacological profile of specific serotonin uptake inhibitor with antidepressan activity. *Prog. Neuropsychopharmac. Biol. Psychiat.* 277–295.

Jaeger C. B., Teitelman G., Joh T. H., Albert V. R., Par D. H. and Reis D. J. (1983) Some neurons of the r central nervous system contain aromatic-L-amino-ac decarboxylase but not monoamines. *Science* **21** 1233–1235.

Jaeger C. B., Ruggiero D. A., Albert V. R., Park D. H., J T. H. and Reis D. J. (1984) Aromatic L-amino ac decarboxylase in the rat brain: immunocytochemi localization in neurons of the brain stem. *Neuroscience* 691–713.

Jones-Villeneuve E. M., McBurney M. W., Rogers K. and Kalnins V. I. (1982) Retinoic acid induces embryo carcinoma cells to differentiate into neurons and gl cells. *J. Cell. Biol.* **94**, 253–262.

Legrand J. (1983) Hormones thyroïdiennes et maturation système nerveux. *J. Physiol., Paris* **78**, 603–652.

Lowry O. H., Rosebrough N. J., Farr A. L. and Rand R. J. (1951) Protein measurement with the Folin phen reagent. *J. biol. Chem.* **193**, 265–275.

McEwen B. S. (1981) Neural gonadal steroid actio *Science* **211**, 1303–1311.

Nimi K. I., Harada I., Kusara Y. and Kishi S. (1962) The ontogenetic development of the diencephalon of the mouse. *Tokushima J. exp. Med.* **8**, 203–238.
Osborne N. N. (1982) *Biology of Serotonergic Transmission*. Wiley, Chichester, Sussex.
Pearse A. G. E. (1969) The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryonic, physiologic and pathologic implications of the concept. *J. Histochem. Cytochem.* **17**, 303–313.
Raff M. C., Abney E. R., Cohen J., Lindsay R. and Noble M. (1983) Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. *J. Neurosci.* **3**, 1289–1300.
Schmechel D. E. (1985) γ-Subunit of the glycolytic enzyme enolase: nonspecific or neuron specific? *Lab. Invest.* **52**, 239–242.
Schmechel D. E., Marangos P. J. and Brightman M. (1978) Neuron-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. *Nature, Lond.* **276**, 834–836.
Schubert D., Lacorbiere M., Whitlock C. and Stallcup W. (1978) Alterations in the surface properties of cells responsive to nerve growth factor. *Nature, Lond.* **273**, 718–723.
Sharp G. A., Shaw G. and Weber K. (1982) Immunoelectronmicroscopical localization of the three neurofilament triplet proteins along neurofilaments of cultured dorsal root ganglion neurones. *Expl Cell Res.* **137**, 403–413.
Shaskan E. G. and Snyder S. H. (1970) Kinetic of serotonin accumulation into slices from rat brain. Relationship to catecholamine uptake. *J. Pharmac. exp. Ther.* **175**, 404–418.
Shimada M. and Nakamura T. (1973) Time of origin in mouse hypothalamic nuclei. *Expl Neurol.* **41**, 163–173.
Solter D., Shevinsky L., Knowles B. B. and Strickland S. (1979) The induction of antigenic changes in a teratocarcinoma stem cell line (F9) by retinoic acid. *Devl Biol.* **70**, 515–521.
Steinbusch H. W. M., Verhofstad A. A. J. and Joosten H. W. J. (1978) Localization of serotonin in the central nervous system by immunohistochemistry: description of a specific and sensitive technique and some applications. *Neuroscience* **3**, 811–819.
Strickland and Mahdavi V. (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* **15**, 393–403.
Teitelman G., Jaeger C. B., Albert V., Joh T. H. and Reis D. J. (1983) Expression of aminoacid decarboxylase in proliferating cells of the neural tube and notochord of developing rat embryo. *J. Neurosci.* **3**, 1379–1388.
Thibault J., Vidal D. and Gros F. (1981) *In vitro* translation of mRNA from rat pheochromocytoma tumors—characterization of tyrosine hydroxylase. *Biochem. biophys. Res. Commun.* **99**, 960–965.
Tillet Y. and Dubois M. P. (1986) Centres sérotoninergiques encéphaliques du mouton: étude critique de la systématisation immunocytochimique. *C.r. Acad. Sci., Paris, D.* In press.
Toran-Alleran C. D. (1981) Gonadal steroïds and brain development. *In vitro* veritas? *Trends in Neurosciences* **4**, 118–121.
Unsicker K., Vey J., Hofmann H. D., Muller T. H. and Wilson A. J. (1984) C6 glioma cell-conditioned medium induces neurite outgrowth and survival of rat chromaffin cells *in vitro*: comparison with the effects of nerve growth factor. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2242–2246.
Voller A., Bidwell D. E. and Bartlett A. (1979) *The Enzyme Linked-Immunoadsorbent Assay* (*ELISA*). Dynatech Eur. Publ., p. 17.
Walicke P. A. and Patterson P. H. (1981) On the role of cyclic nucleotides in the transmitter choice made by cultured sympathetic neurons. *J. Neurosci.* **1**, 333–342.
Wasserman E. and Levine L. (1961). Quantitative microcomplement fixation and its use in the study of antigenic structures by specific antigen–antibody inhibition. *J. Immun.* **87**, 220–245.
Waymire J. K., Gilmer-Waymire K. and Boehme R. (1978) Concomitant elevation of tyrosine hydroxylase and dopamine β-hydroxylase by cyclic AMP in cultured mouse neuroblastoma cells. *J. Neurochem.* **31**, 699–705.
Wong D. T., Bymaster F. P., Reid L. R. and Threlkeld P. G. (1983) Fluoxetine and two other serotonin uptake inhibitors without affinity for neuronal receptors. *Biochem. Pharmac.* **32**, 1287–1293.